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Negative and positive factors determine the activity of the polyoma virus enhancer α domain in undifferentiated and differentiated cell types

(enhancer-binding factors PEA1 and PEA2/embryonal carcinoma cells/fibroblasts/cycloheximide)

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ABSTRACT **The host range of polyoma virus is dependent upon the activity of its enhancer, which is inactive in undifferentiated embryonal carcinoma cells, such as F9 cells, and is active after their differentiation. We show here that the activity of the α domain of the polyoma virus enhancer displays a similar cell-specificity and inducibility as does the whole enhancer. We present evidence to show that its activity is determined by the balance between the activities of two factors, PEA2, a labile repressor, and PEA1, an inducible positive factor that we have characterized previously. Changes in repressor activity help account for the increase in α-domain activity after differentiation of F9 cells. These results suggest that PEA2 is crucial in the regulation of viral gene expression and perhaps more generally in the control of gene expression during differentiation.**

The host range of polyoma virus (Py) is restricted by the cell-type-specific activity of its enhancer (1). The Py enhancer is inactive in embryonal carcinoma (EC) cells, such as F9 cells, and its activity increases after differentiation of these cells (2–7). However, the enhancer is also inactive in some differentiated cell types (8). Negative (9–11) as well as positive factors contribute to its cell-type-specific activity. In addition, its activity is regulated both negatively and positively by oncogene expression (8, 12, 13). It is important to identify the transcription factors that mediate this regulation. The Py enhancer has been subdivided into five domains (14–16). We have found that the activity of the α domain closely parallels that of the whole enhancer in both differentiated and undifferentiated F9 cells (this report; see also refs. 17 and 18). The α domain interacts specifically in cell extracts with two factors, PEA1 and PEA2 (17, 19). PEA1 resembles the transcription factor AP1 and the protein encoded by the oncogene *jun* (17, 20–22). It mediates positive activation of α-domain activity in response to six different transforming oncogenes, the tumor promoter phorbol 12-myristate 13-acetate ("12-tetradecanoylphorbol 13-acetate," TPA), and serum ingredients and during differentiation of F9 cells (17, 23). We present evidence here that PEA2 is a repressor of α-domain activity. Changes in its repressor activity appear to be involved in changes in α-domain activity upon differentiation of F9 cells, but they do not account for induction of α domain activity by oncogenes, TPA, and serum.

MATERIALS AND METHODS

Standard molecular biological techniques were used. The recombinant plasmids shown in Fig. 1 were constructed by ligating synthetic oligonucleotides into the *Xho* I site in the polylinker of the reporter recombinant pG1 (24). The sequences of the inserts were verified. The recombinants were transfected into L-M(TK$^-$) fibroblasts (thymidine kinase-deficient mouse L cells) by the DEAE-dextran/chloroquine technique (8) and into undifferentiated F9 murine EC cells and differentiated F9 cells by calcium phosphate precipitation (8). Total RNA was analyzed by quantitative S1 nuclease mapping followed by scanning of suitably exposed autoradiograms. Transfections were repeated at least four times with two different DNA preparations. F9 cells were induced to differentiate by incubation in 0.1 μM retinoic acid for 4 days before transfection in the presence of retinoic acid.

RESULTS

PEA2 Inhibits PEA1 Activity in L-M(TK$^-$) Fibroblasts. The α domain of the Py enhancer interacts with two specific DNA-binding proteins, PEA1 and PEA2 (refs. 17 and 19; unpublished results). We studied the transcription regulatory activity of these two factors *in vivo* by measuring the effects of oligonucleotides containing their binding sites on transcription from a rabbit β-globin reporter gene. The activities of five recombinants were compared (Fig. 1). pG1P4 contains four head-to-tail copies of oligonucleotides (P) with the sequence of the α domain, upstream from the β-globin promoter. pG1PA4 and pG1PB4 contain oligonucleotides with point mutations that lie exclusively in the binding sites for PEA1 and PEA2, respectively. Studies with cell extracts showed that these mutations prevent binding of the corresponding factors and have no effect on the binding of the adjacent factor (19). pG1PAB4 contains oligonucleotides with mutations in both binding sites, whereas pG1 lacks inserted oligonucleotides.

The reporter recombinants were transfected into L-M(TK$^-$) fibroblasts and, after 24 hr, total RNA was extracted and the amount of specific RNA initiated from the β-globin promoter was measured by quantitative S1 nuclease mapping (see reporter bands in Figs. 2–5). The recombinant pβCBx2 (8) was cotransfected with the reporter recombinants as an internal control for variations in transfection efficiency (see control bands in Figs. 2–5). The experiments were repeated at least four times with two different DNA preparations. The PB multimer, which binds PEA1 but not PEA2, stimulated β-globin transcription 10- to 15-fold (Fig. 2, compare lanes 4 and 1), showing that PEA1 is a positively acting transcription factor. The PA multimer, which binds PEA2 but not PEA1, had no detectable effect on β-globin transcription (lane 3), suggesting that PEA2 is not a positively acting transcription factor. The multimer with the wild-type

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Abbreviations: Py, polyoma virus; EC, embryonal carcinoma; TPA, "12-tetradecanoylphorbol 13-acetate" (phorbol 12-myristate 13-acetate).

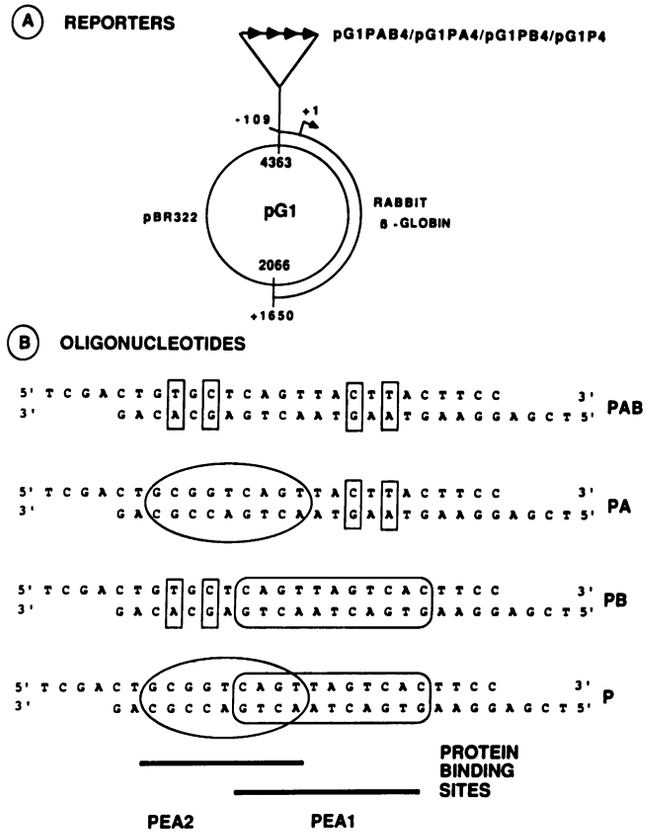

FIG. 1. (*A*) Structure of recombinant plasmids containing protein binding sites of the α domain of the Py enhancer. Numbers outside the circle are nucleotide positions relative to the transcription initiation site (+1); numbers inside the circle are nucleotide positions in the parent plasmid pBR322. The reporter recombinants contain four head-to-tail copies of α-domain oligonucleotides upstream from the rabbit β-globin gene. (*B*) Synthetic α-domain oligonucleotides. The proteins PEA1 and PEA2 protect the circled sequences from digestion by DNase I (17, 19). The mutations (boxed base pairs) prevent binding of either PEA2 (PB), PEA1 (PA), or both factors (PAB).

Py enhancer sequence, with intact binding sites for both factors, stimulated β-globin transcription (lane 5). However, this stimulation was about 5 times lower than that of the multimer that binds only PEA1, showing that binding of PEA2 inhibits PEA1 activity. The PAB multimer, which binds neither factor, did not affect β-globin transcription (lane 2), showing that no other positive factor (that can affect β-globin transcription) interacts with the mutated multimer. These results suggest that binding of PEA2 to its recognition sequence inhibits PEA1 activity in fibroblasts.

Treatment with TPA Does Not Affect Repression. We previously showed that the phorbol ester TPA increases PEA1 activity in fibroblasts (17, 23). We studied whether TPA treatment may also affect the ability of PEA2 to inhibit PEA1 activity. Transfected L-M(TK$^-$) fibroblasts were incubated in low serum for 16 hr and then either treated with TPA or mock-treated for 8 hr (see transfection protocol in Fig. 2). As expected, TPA treatment increased the activity of multimers that can bind PEA1 but not of those with a mutated PEA1 motif (Fig. 2, compare lanes 9 and 10 with lanes 4 and 5 and lanes 6–8 with lanes 1–3). However, the wild-type P multimer was still about 5 times less active than the PB multimer, which binds PEA1, showing that PEA2 still efficiently inhibits PEA1 activity after induction with TPA (compare lanes 9 and 10). These results show that TPA induction of wild-type P-multimer activity does not result from decreased repression of PEA1 activity by PEA2. We

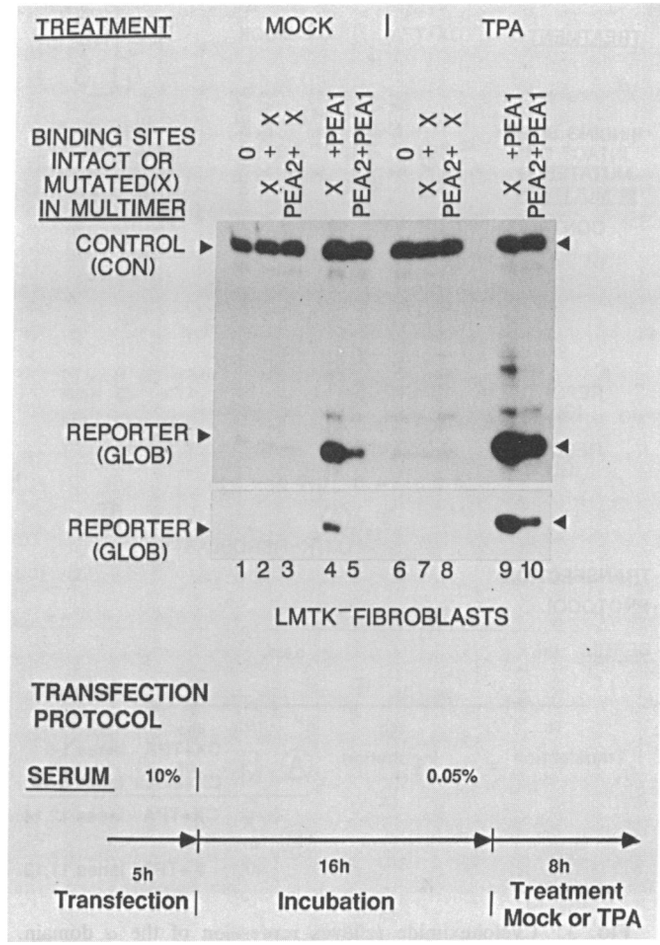

FIG. 2. Activity of the α domain in L-M(TK$^-$) fibroblasts. The cells were transfected with 3.5 μg of the reporter recombinants and 3.5 μg of pβCBx2 (8), incubated overnight in medium containing 0.05% fetal bovine serum, and then incubated for 8 hr in the presence (100 ng/ml) or absence (mock treatment) of TPA as indicated in the diagram of the transfection protocol. Total RNA was extracted and analyzed by quantitative S1 nuclease mapping for RNA initiated from the globin (GLOB) promoter of the reporter recombinants and the conalbumin (CON) promoter of the internal control. A shorter exposure of the reporter bands is shown below the full autoradiogram.

have found in comparable experiments that serum ingredients and *ras* expression stimulate PEA1 without affecting the inhibition by PEA2 (results not shown).

Cycloheximide Treatment Relieves Inhibition by PEA2. Treatment of cells with cycloheximide, a protein-synthesis inhibitor, induces transcription from certain promoters, suggesting that their activity is regulated by labile repressors (for example, see refs. 25 and 26). To test whether PEA2 might be such a labile repressor, transfected L-M(TK$^-$) fibroblasts were incubated for 16 hr in low serum and then treated with cycloheximide for 8½ hr. Half an hour after the addition of cycloheximide, the cells were either treated with TPA or mock-treated (see transfection protocol in Fig. 3). Despite the presence of cycloheximide the activity of the PB multimer, which binds PEA1 but not PEA2, was inducible by TPA, showing that active protein synthesis is not required for activation of PEA1 in these cells (Fig. 3, compare lanes 9 and 4). However, cycloheximide treatment abolished the difference in activity of the P and PB multimers (Fig. 3, compare lanes 9 and 10 and lanes 4 and 5). To show that cycloheximide treatment increases the activity of the wild-type P multimer rather than decreases the activity of the PB multimer, the

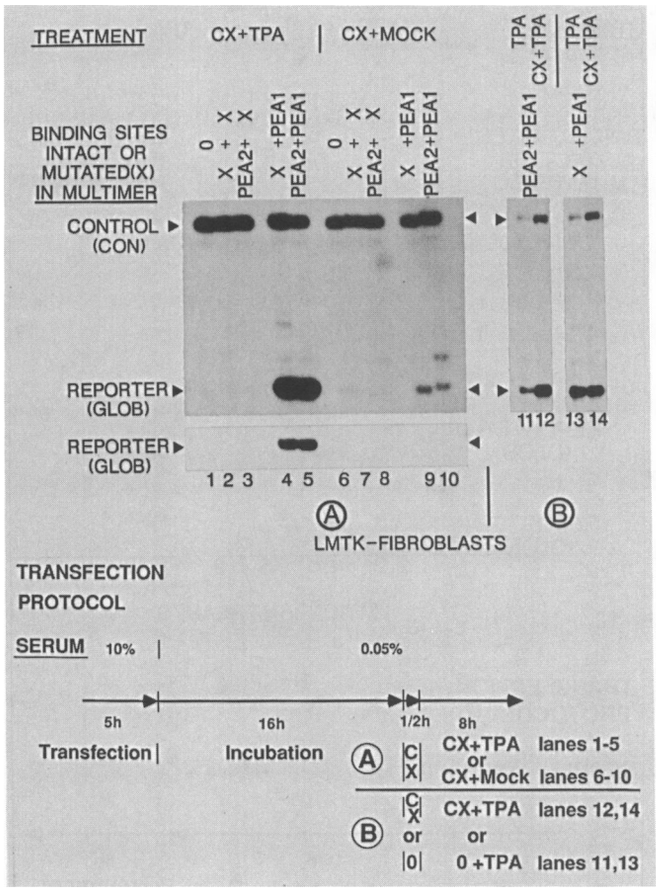

FIG. 3. Cycloheximide relieves repression of the α domain. L-M(TK$^-$) fibroblasts were transfected and incubated in low serum as described in Fig. 2 legend (see transfection protocol). For lanes 1–10, 12, and 14, the cells were then treated for 8½ hr with cycloheximide (CX, 30 μg/ml). Thirty minutes after the addition of cycloheximide they were either treated with TPA (100 μg/ml) (lanes 1–5, 12, and 14) or mock-treated (lanes 6–10). For lanes 11 and 13 the cells were treated with TPA (100 μg/ml) for 8 hr.

activities of the multimers were compared in the presence or absence of cycloheximide and after TPA treatment. The PB multimer, which binds PEA1 but not PEA2, displayed the same activity in the presence or absence of cycloheximide, showing that PEA1 activity does not significantly decrease under these conditions (compare lanes 13 and 14). In contrast, cycloheximide treatment increased the activity of the wild-type multimer about 5- to 10-fold, to the same level as a multimer without a PEA2 binding site (compare lane 12 with lanes 13 and 14). Similar results were obtained in the absence of TPA, showing that the presence of TPA is not required for this lability (results not shown). [We consistently observed an increase in the activity of the cotransfected reporter recombinant upon treatment with cycloheximide, suggesting that this or another labile repressor may also interact with the simian virus 40 enhancer, which is present in this recombinant. A similar activation of simian virus 40 enhancer activity by cycloheximide was observed by Imbra and Karin (27)]. Cycloheximide treatment did not affect the activity of multimers containing mutated PEA1 binding sites (Fig. 3, compare lanes 1–3 and 6–8), showing that the PEA1 motif is necessary for stimulation of β-globin transcription under these conditions. These results suggest that active protein synthesis is required to observe inhibition of α-domain activity and that PEA2 is a labile repressor. They also help to exclude the possibility that there is a positively acting factor that binds to the mutated sequence in the PA multimer but has not been detected in *in vitro* extracts.

Repression Contributes to the Low Activity of the α Domain in F9 EC Cells. The Py enhancer is poorly active in F9 EC cells, possibly due to the presence of repressors that inhibit its activity (9–11). We tested whether the activity of the α domain is repressed in these cells. The wild-type multimer P did not markedly affect β-globin transcription in F9 EC cells (<1.5-fold; Fig. 4, compare lanes 3 and 1). However, the PB multimer, which does not bind PEA2, stimulated β-globin transcription ≈4-fold (compare lanes 7 and 1). Mutation of the PEA1 binding site inhibited this activity (compare lanes 9 and 1; the reporter band is not clearly visible because of the lower level of the internal control band). These results show that the activity of the α domain is repressed in F9 cells.

Repression Decreases upon Differentiation of F9 Cells. The activity of the Py enhancer increases upon differentiation of F9 cells by retinoic acid. This can be accounted for, at least in part, by an increase in the activity (≈5-fold; see ref. 23 and Figs. 4 and 5, lanes 1 and 7) and binding affinity (28) of PEA1. To test whether there was also a decrease in repression, we studied α-domain activity in F9 cells that had been induced to differentiate with retinoic acid. The wild-type multimer P stimulated β-globin transcription about 13-fold (Fig. 5, lanes 1 and 3), about two-thirds the level of stimulation observed with the PB multimer, which has a mutated PEA2 motif (lane 7). These results suggest that there is a decrease in repression upon differentiation. However, the full extent of this difference is difficult to measure because the wild-type multimer does not stimulate β-globin transcription to a significant extent in the undifferentiated cells. To study this possibility further, we stimulated PEA1 activity by *ras* expression. *ras* expression, like TPA treatment (see above), has no effect on repression by PEA2 in L-M(TK$^-$) fibroblasts and in several other cell types (unpublished results). Despite the large increase in PEA1 activity after *ras* expression (Fig. 4, lanes 8 and 7), the wild-type P multimer was still about 5 times less active than the PB multimer in the F9 cells (Fig. 4, lanes 8 and

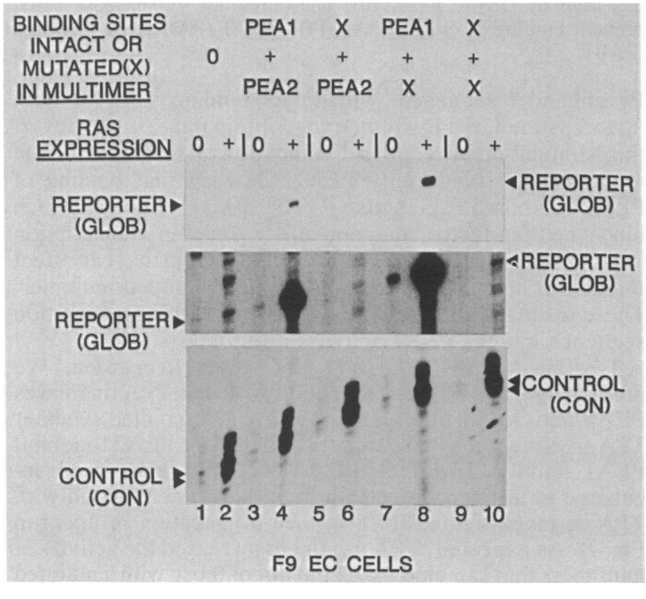

FIG. 4. Activity of the α domain is repressed in undifferentiated F9 EC cells. F9 cells were transfected with 5 μg of reporter recombinant, 9.4 μg of a *ras* expression vector (pRCBx2, + lanes; see ref. 23) or 9.4 μg of a control vector (pΔRCBx2, 0 lanes; see ref. 17). Arrowheads point to specific bands from the globin (GLOB) promoter of the reporter recombinants and the conalbumin (CON) promoter of the cotransfected expression or control vectors. The top panel is a shorter exposure of the middle panel.

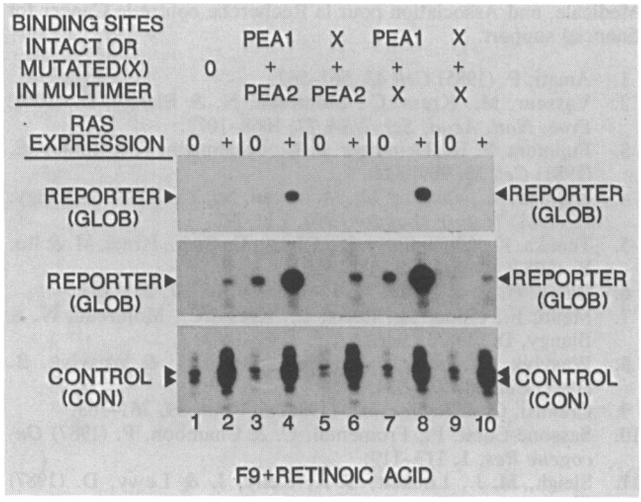

FIG. 5. Activity of the α domain increases after differentiation of F9 cells. Differentiated F9 cells were transfected with 2 μg of reporter DNA and 3.7 μg of *ras* expression vector (pRCBx2, + lanes; ref. 23) or control vectors (pΔRCBx2, 0 lanes; ref. 17). Arrowheads point to specific bands from the globin (GLOB) promoter of the reporter recombinants and the conalbumin (CON) promoter of the expression or control vectors. The top panel is a shorter exposure of the middle panel.

4). [The difference in the control signal in the + ras lanes and the 0 ras lanes was reproducibly observed and probably resulted from differences in the stability of the RNA transcribed from the expression vector and the nonexpressing control vector (8).] In the differentiated cells, this difference was clearly smaller, about 1.5-fold (Fig. 5, lanes 8 and 4). In addition to repression of multimer activity, there was another difference between repressor activity in the EC cells and repressor activity in their differentiated counterpart. The PA multimer, which contains a mutated PEA1 binding site, inhibited the β-globin promoter in the parent cells (Fig. 4, lanes 5 and 1) but not in the differentiated cells (Fig. 5, lanes 5 and 1; the bands were clearly seen on the original autoradiograms). This difference in the behavior of the PA multimer was also observed after *ras* expression (Figs. 4 and 5, lanes 6 and 2; the small increase in activity in lane 6 compared to lanes 2 and 10 in Fig. 5 was not observed in other experiments). Mutation of the PEA2 motif decreased β-globin repression (Figs. 4 and 5, compare lanes 1 and 10), showing that repression is mediated by the PEA2 binding site. These results further suggest that repressor activity decreases during differentiation of F9 cells.

DISCUSSION

Roles of PEA1 and PEA2 in Control of α-Domain Activity in Fibroblasts. We previously showed that α-domain activity is positively regulated by a number of agents in fibroblasts, including transforming oncogenes, a tumor promoter, and serum growth factors (17, 18, 23). We now present evidence that suggests that the activity of this domain is also negatively regulated. However, relief of this repression is most probably not involved in induction of α-domain activity by these agents. This conclusion is supported by the following observations. (*i*) Experiments using *in vitro* extracts suggest that there is no cooperativity in the binding of PEA1 and PEA2 to the α domain, and thus that they can be considered as separate entities in terms of regulation of their individual activities (17, 19). (*ii*) The activity of the α domain is inducible to a similar extent *in vivo*, whether or not the PEA2 binding site is intact. However, an intact PEA1 binding site is absolutely required (refs. 17 and 23 and this study). Repression is still observed after induction of α-domain activity. (*iii*) Repression is decreased by treatment with cycloheximide. Under these conditions, the activity of the α domain is still inducible by TPA. (*iv*) The activity of the α domain is inducible by oncogene expression in cell lines that contain low levels of both repressor activity and PEA2 protein in cell-free extracts [differentiated F9 cells (this study and ref. 28); myeloma cells (unpublished results)]. (*v*) The activity of oligonucleotides with the PEA1/AP1 binding sequences of the metallothionein and simian virus 40 enhancers is also inducible by *ras* expression (unpublished results), even though the only obvious sequence similarity between their sequences and the α domain is in the PEA1/AP1 motif. These results show that induction of α-domain activity is most probably mediated by an increase in PEA1 activity, rather than by a decrease in repression by PEA2.

PEA1 activity is stimulated by TPA in the presence of cycloheximide in L-M(TK$^-$) cells. In contrast, in myeloma cells, cycloheximide blocks the stimulation by TPA (18). These results suggest that activation of PEA1 activity by TPA depends upon two events, one of which requires active protein synthesis. In high serum (10% fetal bovine serum) L-M(TK$^-$) cells contain high levels of PEA1 activity, and myeloma cells contain low levels (23). Active protein synthesis may in fact be required to produce PEA1 itself, since this step of activation is critical (i.e., cycloheximide-sensitive) in the cells containing low levels of this factor. However, it is also possible that the synthesis of other proteins, such as the *fos* gene product, are required to activate PEA1 (see ref. 23).

The Balance Between Negative and Positive Activities, as well as Their Absolute Levels, Determines α-Domain Activity in Different Cell Types. The α domain is not detectably active in F9 cells. The simplest interpretation of the results we present here is that there are positive and negative factors that interact with this domain in these cells. Thus, the α domain is inactive not because there is a total lack of positive factors that interact with this domain, but because PEA1 and PEA2 (or similar factors) are present in F9 cells, and repression by PEA2 inhibits PEA1 activity. Repression of a positively acting factor contributes to the low activity of the domain. After differentiation there is a large increase in α-domain activity. The effects of mutations in the PEA1 and PEA2 binding sites suggest that this results from a decrease in repression as well as an increase in the activity of a positive factor. It is striking that the α domain is more active in fibroblasts than in differentiated F9 cells, even though its activity is clearly repressed. This suggests that the balance between negative and positive activities, as well as their absolute amounts, determines the final activity of the α domain.

Our results contrast with those of several other studies. Sleigh *et al.* (11) used competition studies to show that the A domain of the Py enhancer, which encompasses the α and δ domains, interacts with a repressor in F9 EC cells but not in differentiated F9 cells. These results agree with ours. However, they did not detect repression in Ltk$^-$ fibroblasts. Their competition assays were performed with large fragments, which bind many factors and could titrate several positive and negative factors at the same time. Under these conditions a repressor activity in Ltk$^-$ cells could easily have been missed. Kryszke *et al.* (28) showed, by using a gel retardation assay with cell extracts, that there is a low level of PEA1 DNA-binding activity in F9 EC cells and that this level increases after differentiation. However, the final level of DNA-binding activity is still lower than in fibroblasts. These *in vitro* results for PEA1 agree with our *in vivo* studies and indicate that PEA1 is most probably responsible for the activity of the PB multimer in the EC cells and for the increased activity of the multimers that we observe upon

differentiation of F9 cells. In addition, we have found a good correlation between the *in vivo* activities of multimers and the levels of DNA-binding activities of PEA1 (and of PEA2) in extracts from myeloma cells (unpublished results). However, Kryszke *et al.* (28) did not detect PEA2 in F9 EC cell extracts. The inhibitory activity we observe in these cells is decreased by mutation of the PEA2 binding site. It should be recalled that the assay they used to detect PEA2, DNase I protection ("footprinting") also did not detect PEA1, even though it is present in these extracts, as shown by their gel-retardation experiments. The apparent lability of PEA2 (as revealed in the presence of cycloheximide) might contribute to difficulties in detection of this factor in some cell extracts. However, our interpretations of the transfection data depend upon comparisons with *in vitro* and *in vivo* data from other cell types, and further studies will be required to show that PEA2 is present in F9 cells and that its activity decreases during differentiation.

CONCLUSION

Our results strongly suggest that the overall transcriptional activity of the α domain of the Py enhancer is determined by the balance between the positive and negative effects of the transcription factors PEA1 and PEA2, respectively. However, it should be emphasized that this is the simplest interpretation of our *in vivo* results and depends upon the parallels we have drawn with *in vitro* data (17, 19). We cannot absolutely exclude that other factors exist *in vivo* that are not detected *in vitro*, and further studies using functional assays *in vitro* and footprinting experiments *in vivo* would help to support our model. In other systems, comparable balances between negative and positive activities appear to exist. For example, compensating negative and positive activities probably result in the inactivity of the immunoglobulin heavy-chain enhancer in nonspecific cells (24, 29). Both negative and positive factors are involved in the induction of β-interferon gene transcription (30).

The transcription factors PEA1 and PEA2 are probably involved in the regulation of other transcription elements. PEA1 is involved in the regulation of transcription of a number of genes in response to oncogenes, TPA, and growth factors (17, 23). Less is known about the role of PEA2. Comparison of footprint patterns suggests that it is not related to the recently described simian virus 40 enhancer factor(s) AP4 or GTIIA, which bind to a sequence adjacent to the PEA2 binding site in the Py enhancer (31, 32). However, our observations that PEA2 repressor activity is labile, changes during differentiation, and represses PEA1 (AP1) activity suggest that PEA2 is important for regulation of gene expression.

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